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(54) Title: ARTIFICIAL SKIN CONTAINING AS SUPPORT BIOCOMPATIBLE MATERIALS BASED ON HYALURONIC ACID DERIVATIVES (57) Abstract Artificial human skin comprising: a) microperforated membrane based on a hyaluronic acid derivative, on which keratinocytes have been seeded and cultured, b) an underlying non-woven tissue based on a hyaluronic acid derivative wherein fibroblasts have been seeded and left to proliferate. The artificial human skin according to the present invention can be therefore advantageously used in medicine, in surgery, in diagnostics and as a vehicle for preparing a controlled release medicament. Lastly, this artificial human skin can be frozen to -80 °C or placed in liquid nitrogen and stored, so that a tissue bank can be created.		

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ARTIFICIAL SKIN CONTAINING AS SUPPORT BIOCOMPATIBLE MATERIALS BASED
ON HYALURONIC ACID DERIVATIVES

FIELD OF THE INVENTION

The present invention relates to:

- 5 - an artificial human skin constituted by a completely differentiated epidermis and dermal appendage, and containing as the support two biocompatible materials based on hyaluronic acid (HA) derivatives
- the processes for preparing this artificial human skin,
- 10 - the use of this artificial skin in medicine and in surgery, as a diagnostic device, and as vehicling agent for preparing controlled release medicament.

BACKGROUND OF THE INVENTION

Skin loss due to trauma or disease is usually treated by the autograft
15 technique, that is, by substituting the missing skin with pieces taken from donor areas of the same patient. An important step forward in the treatment of such lesions by reconstructive surgery is represented by *in vitro* cultures of keratinocytes (kc) (J. Rheinwald and H. Green, Cell, 6:331, 1975), whereby said cultures are allowed to expand *in*
20 *vitro*, and membranes of epidermal cells are obtained which are potentially useful to cover skin wounds. This technique has been widely used in clinical practice, mainly for burn patients (G. G. Gallico et al., M. Engl.J. Med., 311-448, 1984), but problems arose right from the start, such as the difficulty for such grafts to take,
25 the fragility of the epithelial sheets and consequent difficulty for the surgeon to handle them.

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A different approach was adopted by Yannas et al. (Science, 215:174, 1982), who used resorbable porous materials constituted by coprecipitates of collagen and glycosaminoglycans (GAG), particularly chondroitin-6-sulphate, covered by a thin film of silicon membrane.

5 The characteristic of such materials is that they present randomly formed, intercommunicating pores, rather like a sponge.

S. Boyce and J. Hansbrough (Surgery, 103-421, 1988) described growing kc on the surface of membranes made of collagen and GAG, reducing the surface porosity of the material. In order to limit the development of
10 epidermal culture on the membrane surface, an additional, non-porous layer was inserted.

Skin graft technology must take into consideration the interaction between kc, the basal membrane and the underlying dermis. Nowadays it is generally thought that in the case of full-thickness lesions,
15 autografts can be notably facilitated by placing a dermal bed in the wound underneath the epithelial layer.

The basal kc thus lie on a more physiological substrate and can develop a basal membrane and dermal-epidermal linking structures, capable of lending the necessary resistance to the graft.

20 Recent clinical studies have suggested that Cuono's method (Langdon et al. J. Invest. Dermatol. 91 5: 478, 1988), whereby a "full-thickness" wound is treated with an allograft from the skin of a cadaver, gives better results in terms of the percentage of graft which takes and general quality of the skin on healing.

25 However, grafts are difficult to obtain, expensive to store and are potential carriers of pathogenic viruses.

There is clearly a need for new, biodegradable, artificial skin

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substitutes which do not have these drawbacks, and satisfy the following requirements:

- 1) their surfaces must allow for adhesion and cell growth;
- 2) neither the polymers themselves, nor their degradation products
- 5 should cause inflammation or toxic phenomena when implanted *in vivo*;
- 3) the product should be perfectly reproducible in its three dimensions;
- 4) its ideal porosity is 50%, which gives a large surface area for cell-polymer interactions, sufficient volume for the deposit of
- 10 extracellular matrix and only slight, or no, migrational impediments during *in vitro* culture.
- 5) the supporting polymer should be absorbed once the regenerated tissue no longer requires it. Indeed, foreign bodies *in vivo* represent a high risk of infection and/or inflammation.
- 15 Indeed, the products already on the market or being developed present certain drawbacks: their degradation is uncontrollable and interferes with the wound healing process, thus favouring inflammation. Moreover, these substitutes require the epithelial cells to be thickly seeded on the support and left to proliferate for a long time.
- 20 Some examples of the products known to date and generally recommended for use in treatment of severe burns are:
 - 1) Dermagraft[®], developed by ATS (California), wherein heterologous human fibroblasts are cultivated on a spongy, resorbable material constituted by polylactic, polyglycol or polygalactoside acid.
 - 25 Autologous kc are seeded onto these materials;
 - 2) GraftSkin[®], produced by Organogenesis Inc. (Boston, USA), wherein heterologous human fibroblasts are cultivated on a collagen based

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substrate;

3) AlloDerm[®], produced by Life Cell Corp. (Texas, USA) and based on human or pigs' skin, wherein the basal membrane and dermal matrix remain intact. The tissue is stored at a low temperature (-80°C) until
5 ready to be used, when it is seeded with autologous fibroblasts and kc and then grafted into the patient.

However, these, and other products do not allow the *in vitro* reconstruction of a perfectly functional dermoepidermal junction.

The use of hyaluronic acid (HA) derivatives has also been described
10 (EPA. 0216453) for the preparation of suitable products, particularly membranes to support human kc growth (EPA 0462426) and non-woven tissues (WO 93/11803). Abatangelo et al. (Wound Repair and Regeneration, January-March, p. 80, 1995) reported the use of nonwoven tissue to support mixed cultures of human kc/fibroblasts. Even so, the
15 epithelial layer was not homogeneous because of the kc which seeded in the gaps in the non-woven tissue, thus giving it an uneven thickness. Degradation of the polymers considered here mainly produces hyaluronic acid (HA), which is a normal constituent of the extracellular matrix and therefore has the advantage of being metabolized by normal cell
20 mechanisms.

The chemical modifications allow the product to remain at the site of the graft for far longer than is possible with the natural polymer, and the receptor interactions are maintained throughout that time. In this regard, it must be remembered that the main receptor for HA,
25 known as CD44, is normally expressed in the epithelial tissues, particularly in the basal and spinous layers of the epidermis, while expression of the protein gradually decreases in the upper layers

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until it disappears in the completely differentiated kc (Carter et al., J. Cell Biol. 113:207, 1991). Moreover, the role of CD44 in HA's degradation mechanism is well documented (Culty et al. J. Cell. Biol. 111:2765, 1990; Underhill, Dev. Biol. 155:324, 1993).

- 5 The purpose of the present invention is an artificial human skin simulating both the epidermal and dermal layer of the natural one, wherein both fibroblasts and keratinocytes (kc) are present, both cell types actively proliferating and separated, at the interface, by a protein extracellular matrix, having the characteristic of a
- 10 dermoepidermal junction.

SUMMARY OF THE INVENTION

The Applicant has therefore unexpectedly found the artificial human skin subject of the present invention which comprises:

- a) a microperforated membrane based on a hyaluronic acid derivative,
- 15 on which keratinocytes have been seeded and cultured,
- b) an underlying non-woven tissue based on a hyaluronic acid derivative wherein fibroblasts have been seeded and left to proliferate.

- The artificial skin, subject of the present invention generally
- 20 further comprises an extracellular protein matrix containing proteins of the dermo-epidermal junction, placed at the interface between keratinocytes and fibroblasts.

- Thanks to its biodegradability, which can also be a result of the receptor mechanism mentioned previously, the artificial human skin is
- 25 spontaneously absorbed within a set time, leaving just the newly-formed tissue at the lesion site.

Therefore the artificial human skin according to the present invention

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can be advantageously used in medicine and in surgery.

In addition the artificial human skin can be advantageously used as a vehicle for preparing controlled release medicaments containing at least one active principle selected from the group consisting of
5 biologically active compounds, proteins, peptides and mixtures thereof, wherein said active principle is adsorbed to the fibres of the non-woven tissues and/or to the microperforated membrane.

Moreover, it is possible to use artificial skin as a diagnostic device for *in vitro* tests to verify the possible toxic effect of products
10 and/or substances destined to be placed in contact with the skin and to be used in the chemical, pharmaceutical, cosmetic and agricultural fields.

The artificial skin can be also advantageously used in the cosmetic field in particular for hair graft.

15 In fact dermal hair follicle fibroblasts can be used along with keratinocytes isolated from the same region in order to build up an organotypic culture showing features of the "in vivo" site of origin as described in "Human Hair Follicle Germinative Epidermal Cell Culture"

20 Amanda J. Reynolds et al. Rapid Communication pp. 634-638.

Lastly, a further advantage of the artificial human skin is that it can be frozen to -80°C or placed in liquid nitrogen and stored, so that a tissue bank can be created, thus enabling immediate grafts whenever and wherever needed, as the product can thus be constantly
25 available, even in centres which are not specialized in dermoepidermal cultures.

A further object of the present invention resides in the processes for

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preparing said human artificial skin.

In particular the first process comprises the following steps:

- i) seeding keratinocytes cultures at a density ranging from 1.000 to 100.000 cell/cm², on the microperforated membranes and expanding the Kc on said membrane. The conventional culture technique involving the use of Foetal Calf Serum (FCS) is followed until partial or complete confluence is achieved.
- ii) cultivating the fibroblasts isolated from the dermis or from other districts by the usual techniques in DMEM containing 10% foetal calf serum, conditioning these fibroblasts to proliferate first by seeding them on plastic, and then seeding the fibroblasts in the non-woven tissue and continuing the culture until freezing, (in case the obtained artificial skin must be preserved) or until grafting.
- iii) laying the epithelial layer formed on the microperforated membrane on the non-woven tissue colonized by fibroblasts, being careful that the outer edge of the microperforated membrane does not touch the bottom of the dish and that it extends slightly beyond the underlying non-woven tissue,
- iv) optionally fixing together the epithelial cells layer on the microperforated membrane to the underlying non-woven tissue colonized by fibroblasts by means of biological adhesives selected from the group consisting of: collagen, fibrin, fibrin glue.
- v) adding a sufficient volume of the same medium used in step (i), keeping the culture immersed so that the keratinocytes of the upper layer of the microperforated membrane are at the air-liquid interface, adding at the first and subsequent changes of medium, ascorbic acid at concentration equal to 1µg/ml, continuing the culture until

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grafting or freezing.

It is known that keratinocytes can be cultivated in medium free from foetal calf serum and feeder-layer of murine fibroblasts, so the artificial skin, which is the subject of the present invention, can be
5 obtained by the simultaneous seeding of human fibroblasts in the non-woven tissue and of keratinocytes on the membrane. In this case a "chemically defined" medium is used which is suitable, for keratinocyte growth. In this case the process comprises the following steps:

- 10 i') seeding the fibroblasts in the non-woven tissue and continuing the culture until freezing or grafting.
- ii') laying the microperforated membrane on the non-woven tissue, with its edges extending slightly beyond those of the underlying tissue and fixing said membrane with the biological adhesives used in the
15 previous process or by mechanical means.
- iii') seeding the keratinocytes on the microperforated membrane, expanding said keratinocytes by using said "chemically defined" medium, which is suitable for keratinocyte growth on plastic dishes until confluence is achieved.
- 20 iv') adding a sufficient volume of the medium used in the preceding step, keeping the culture immersed so that the keratinocytes of the upper layer of the microperforated membrane are on the air-liquid interface, adding at the first and subsequent changes of medium, ascorbic acid at concentration equal to 1µg/ml, continuing the
25 culture until grafting.

The above processes according to the present invention comprise a further step consisting of cryopreserving said artificial skin

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in the presence of a cryopreserving agent, when the skin must be preserved.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows Hematoxylin and Eosin staining of a 15-day-old composite
5 dermal-epidermal culture. Kcs have migrated through the Laserskin^R membrane to the non-woven tissue, which is embedded with fibroblast cells (x100).

Fig. 2 shows the immunohistochemistry of the composite culture probed with an antiinvolucrin antibody, the outer epithelial layer is
10 undergoing keratinization (x 200).

Fig. 3 shows the immunohistochemistry of the composite culture probed with anti-KL4 (total keratin) antibody, a classic Kc marker (x 200).

Fig. 4a shows the immunohistochemistry of the composite culture showing the $\beta 4$ integrin subunits expression, predominantly found in
15 the basal Kc cell layer (x 100).

Fig. 4b shows the immunohistochemistry of the composite culture showing the $\beta 1$ integrin subunit mainly expressed at the intercellular bridges joining the epithelial cells (x 100).

Fig. 5 shows the immunolocalization of basal Kcs expressing the CD44
20 receptor (x 200).

Fig. 6 shows the immunohistochemistry of the composite culture showing the basement-membrane-like zone, as can be seen from the extent of laminin deposition (x 200).

DETAILED DESCRIPTION OF THE INVENTION

25 The epithelial layer is reconstructed by seeding human kc on a microperforated membrane, prepared according to USP 5,326,356, which we incorporate by reference, while the human fibroblasts are

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cultivated on a material known as non-woven tissue, as described in the International Patent Application No. WO 93/11803, which we incorporate herewith by reference.

The hyaluronic acid derivative used for preparing both the
5 microperforated membrane and the non-woven fabric is preferably an ester of hyaluronic acid. Preferably these esters have a percentage of esterification comprised between 75% and 100%. By varying the percentage of esterification of the hyaluronic acid esters used, it is possible to control the degradation kinetics of the tissue support
10 and, therefore, the length of time that the device can remain in situ. The preferred hyaluronic acid esters are the benzylesters of hyaluronic acid.

The microperforated membranes of hyaluronic benzylester are in particular already commercially available with the trademark
15 Laserskin®

Both fibroblasts and keratinocytes are of human origin or of a species which is compatible when grafted into humans.

When both these kinds of cells are of human origin they can be heterologous, autologous or combinations of these two types.

20 As previously pointed out the human artificial skin subject of the present invention can be advantageously used in medicine and surgery.

In fact the final outcome of the wound healing process is considerably improved, with particular reference to cases of:

- 25
- 1) deep, II and III-degree burns.
 - 2) various kinds of ulcer: diabetic, venous, bedsores;
 - 3) plastic surgery.

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Indeed, a physiological covering for these kinds of lesions, albeit composed of heterologous cells, reduces the risk of infection or excessive loss of organic fluids. Moreover, the proliferating cells in the dermal bed secrete substances (still partly unknown) such as growth factors and cytokines, which accelerate the wound healing process.

Moreover the skin according to the present invention can be used for the treatment of diseases characterized by melanine deficiency such as vitiligo.

10 In this case the artificial human skin of the present invention contains a quota of melanocytes which can be grafted by means of the same skin to patients affected by vitiligo.

The artificial human skin can in particular also be applied in genetic therapy, wherein the cells of a patient (kc, fibroblasts or both) are genetically modified, for example to correct congenital malformations and/or metabolic defects.

When the human artificial skin according to the present invention is used as vehicling agent for preparing controlled release medicaments containing proteins, these are preferably growth factors, such as FGF (Fibroblast Growth Factors), TGF β (Transforming Growth Factor β), KGF (Keratinocyte Growth Factor), NGF (Nerve Growth Factor), proteins involved in the clotting cascade such as Coagulation Factor VIII and others. Moreover, proteins of the extracellular matrix, such as Fibronectin, Laminin, Collagen can be adsorbed to the fibres of the non-woven tissue, to improve the cell attachment process.

In the first process according to the present invention, in step (i) the seeding density of keratinocytes cells is preferably comprised

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between 5,000 and 25,000 cells/cm².

The conventional technique used for expanding these keratinocytes and involving the use of foetal calf serum are basically those described by Rheinwald and Green (Cell, 6:331, 1975). In particular CEC culture
5 medium is used (Green H. et al., J. Proc. Nation. Acad. Sci. 76:5665, 1979), in the presence of a "feeder-layer" of nonproliferating 3T3-J2 murine fibroblasts. The mean confluence time is generally comprised between 7-12 days or 15-24 days from the initial setting up of the culture.

10 In step (i') of the second process the commercially available medium called MCDB 153 (or others) is preferably used as "chemically defined" medium for expanding the keratinocyte cells.

According to the present invention in both the processes primary or defrozen cultures of fibroblasts and keratinocytes can be used.

15 The cryopreservation step used in both processes for preparing the artificial human skin, when the same must be preserved, is preferably carried out in the presence of dimethylsulfoxide or glycerol as cryopreserving agents.

It is possible to demonstrate that in the artificial human skin
20 according to the present invention kc migrate through the micropores in the membrane, to the upper part of the non-woven tissue in direct contact with the membrane, while the fibroblasts produce a considerable quantity of protein extracellular matrix.

Of considerable interest is also the fact that kc embedded in the non-
25 woven tissue and in contact with the fibroblasts, take on the appearance of epithelioid basal cells, expressing membrane markers typical of the cells in the basal layer of the epidermis. Of these, we

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examined the expression of CD44, and found that the protein is localized in the innermost basal layers of the composite and, above all, in the epithelial cells in direct contact with Hyaff 11 (benzyl ester of HA), both in the form of non-woven tissue and in the form of microperforated membranes. This suggests that, besides acting as a support for the cells, the biomaterial also interacts with the cells which adhere to it.

Lastly, it is possible to observe the production of specific proteins of the dermoepidermal junction (laminin, collagen, types III, IV and VII), while on the "dermal" side there is abundant production of the extracellular matrix, shown by specific staining and immunohistological methods (see example 1).

For purely indicative purposes, and without being limited by the same, we report here some examples characterising the new-formed tissue.

Example 1

A piece of non-woven tissue (1.5 x 1.5 cm) constituted by Hyaff 11 (benzyl ester of HA) is laid on the bottom of a culture dish and fixed in place at the four corners with fibrin glue. Primary human fibroblasts are seeded on the non-woven tissue (0.1×10^6 cells in 0.2 ml of medium) slowly soaking the non-woven tissue. The dish is covered and left to stand for about 30 minutes under a sterile hood, after which the volume is brought to 2 ml with DMEM containing 10% FCS and the dish is incubated at 37°C in an atmosphere of 5% CO₂.

Human kc between the II and IV culture passages are seeded on a Laserskin^R membrane measuring 5 x 5 cm² (Andreassi et al. Wounds 3:116, 1991). The kc can be cultivated in CEC medium (Rheiwald and Green, Cell, 6:331, 1975) in the presence or in the absence of a

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feeder-layer but using a chemically defined medium (Andreassi et al. Wounds, 3:116, 1991). Both in fibroblast cultures and in kc cultures, the relative media are changed every 48-72 hours.

Once a well-stratified epithelial layer has been formed, the
5 Laserskin[®] membrane is cut with a scalpel in sterile conditions, into pieces measuring 1.5 x 1.5 cm. The fibroblast culture on the non-woven tissue is laid on a sterile steel net (about 3 mm high); this is then placed in a Petri dish measuring 6 cm in diameter. The epithelial
10 layer is laid on the non-woven tissue, taking care that no air bubbles are trapped in the interface between the two materials (in the example reported here, no adhesives were used to fix the epithelioid membrane to the non-woven tissue).

A medium such as CEC or MCDB 153, suitable for kc growth, is added in a volume which brings the level of the liquid to just below the upper
15 surface of the epithelial layer, but completely soaking the non-woven tissue.

The medium is changed every 72 hours. The first change of medium is supplemented with ascorbic acid (1 µg/ml). to favour the production of the extracellular matrix. The emergence of the culture is to mimic
20 physiological skin conditions by stimulating complete differentiation of the epithelial cells, as amply reported in the literature (Prunieras et al., J. Invest. Dermatol., 81: 280, 1983; Bernstam et al. *in vitro* Cell. Biol., 22: 695, 1986). The culture is maintained for 14 days, during which time it is observed by phase-contrast
25 microscopy to check that the epithelial layer expands beyond the edges of the membrane. The material's opacity makes any further observation difficult. At the end of the culture time, the composite material is

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cut in half with a scalpel, being careful to maintain its structure. One half is treated by traditional histological techniques (hematoxylin-eosin staining), while the other half is immersed in OCT (medium for the cryopreservation of tissues, Milestones, USA), frozen
5 in liquid nitrogen and then stored at -80°C. The latter is cut into 6 µm thick slices with a cryostat. The slices are used for immunohistochemical investigation, using antibodies against the markers of the differentiation status of the kc, of the dermal-epidermal junction and of the extracellular matrix.

10 Results of histological hematoxylin-eosin staining

Figure 1 (x100) shows a well stratified epithelium over a microperforated Laserskin^R membrane. The upper and outer layer is undergoing keratinization. The kc appear to be well distributed on the underside of the membrane, and to have colonized the underlying
15 nonwoven tissue to a considerable extent. The epithelial layer is very compact with a variable thickness which is never less than 10-15 cells. The dividing line between the layer of epithelial cells and underlying fibroblasts can be seen clearly. The cross-sections of fibres from the non-woven tissue are also clearly visible, as is the
20 Laserskin^R microperforated membrane.

Immunohistochemical characterization

Studies on the tissue markers were conducted with the following antibodies:

- 1) anti-keratin antibody KL4 (Dermatology Department, Immacolata,
25 Rome)
- 2) anti-involucrin polyclonal antibody (Dermatology Department, Immacolata, Rome)

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- 3) anti-human integrin monoclonal antibody $\beta 4$ (Dermatology Department, *Immacolata*, Rome)
- 4) anti-human integrin monoclonal antibody $\beta 1$ (Dermatology Department, *Immacolata*, Rome)
- 5) anti-human CD44 monoclonal antibody (Prof. Abatangelo, Department of Histology and Embryology of the University of Padua)
- 6) anti-collagen polyclonal antibody III (Chemicon, CA USA)
- 7) anti-collagen polyclonal antibody IV (Sigma)
- 8) anti-laminin polyclonal antibody (Sigma)
- 9) anti-fibronectin polyclonal antibody (Sigma)

Immunohistological investigation reveals that:

- a) the outer epithelial layer is well differentiated and undergoing keratinization, as the immunopositivity to involucrin is very marked (fig. 2, x200).
- b) the compact cell layer on the upper side of the non-woven tissue is constituted by kc, since the response to cytokeratin is very strong here compared to the underlying fibroblastic component (Fig. 3, x200);
- c) the subunits $\beta 4$ and $\beta 1$ of the integrins are present in what is essentially a basal layer of the newly-formed tissue. These subunits, and especially $\beta 4$, are expressed in the epidermis, at the level of the basement membrane, in the structures anchoring the membrane to the underlying dermis (hemidesmosomes). In the "artificial skin" depicted here, the positive $\beta 4$ cells (Fig. 4a x100) express the protein predominantly in the basal part, while the integrin $\beta 1$ is expressed in the intercellular bridges joining the kc (Fig. 4b, x100). The observations reported here constitute an important indication of the correct "polarization" of the newly formed epidermis.

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- d) The kc in direct contact with the fibers of the non-woven tissue, like the cells in contact with the top and underside of the Laserskin[®], show a marked immunopositivity to CD44 (Fig. 5, x200). It is thought that this represents a singular feature of the composite culture described here: indeed, CD44 can mediate cell adhesion to the material (constituted by modified hyaluronic acid), and likewise its degradation.
- e) In the basement membrane, and therefore also the dermoepidermal junction, a typical extracellular matrix can be observed, the components of which are mainly produced by the basal kc resident in the area. Characteristic markers are therefore laminin and collagen types IV and VII. Confirmation of these observations is given by the fact that the three components are expressed in the interposition between the basal layer of the kc embedded in the non-woven tissue and the underlying fibroblasts. In particular, the extent of the response to laminin (Fig. 6, x200) is focalized on that which can be considered an "artificial basement membrane".
- f) Lastly, on the side of the dermis, a strong immunopositivity to fibronectin can be found. Fibronectin is a protein of the matrix produced by the fibroblasts which are obviously abundant in the dermis. The lack of response in the epidermal layer again indicated that the formation of a well-structured dermal tissue with the typical natural skin structure has been artificially induced.

Example 2

- 25 In order to demonstrate the possibility of cryopreserving the artificial skin thus produced, the dermal-epidermal cultures are frozen in the presence of a cryopreserving agent such as

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dimethylsulfoxide (DMSO), according to the usual technique. In short, the culture is lifted and placed on a petri dish measuring 10 cm in diameter and containing 20 ml of freezing medium for (45% DMEM, 45% FCS, 10% DMSO) cooled to 4°C. It is left to reach equilibrium for
5 about 5 minutes, then the dish is brought to a temperature of -80°C by a process of continuous freezing at a rate of -1°C/min., starting from 4°C.

After one week the "artificial skin" is defrozen, warmed rapidly to 37°C and the culture is repeatedly washed with DMEM containing 10%
10 FCS, to completely eliminate any residue DMSO. It is left to stand in an incubator at 37°C for 24 hours (in an atmosphere of 5% CO₂), after which the tissue undergoes the histological and immunohistological examination described in Example 1.

Results

15 No significant morphological and/or structural alterations were observed in the material, compared to before freezing.

These results indicate that the artificial skin described herein can be used to constitute "tissue banks", thanks to its resistance to freezing.

CLAIMS

- 1 1. Artificial human skin comprising:
 - 2 a) a microperforated membrane based on a hyaluronic acid derivative,
 - 3 on which keratinocytes have been seeded and cultured,
 - 4 b) an underlying non-woven tissue based on a hyaluronic acid
 - 5 derivative wherein fibroblasts have been seeded and left to
 - 6 proliferate.
- 1 2. The artificial human skin according to claim 1 further comprising
- 2 an extracellular protein matrix, containing proteins of the
- 3 dermoepidermal junction placed at the interface between fibroblasts and
- 4 keratinocytes cells.
- 1 3. The artificial human skin according to one of the claims 1 and 2
- 2 wherein said hyaluronic acid derivative used for preparing both the
- 3 microperforated membrane and the non-woven tissue is an ester of
- 4 hyaluronic acid.
- 1 4. The artificial human skin according to claim 3 wherein said
- 2 hyaluronic acid ester has a percentage of esterification comprised
- 3 between 75% and 100%.
- 1 5. The artificial human skin according to claims 3 and 4 wherein said
- 2 ester is the hyaluronic benzyl ester.
- 1 6. The artificial human skin according to one of the claims 1-5, for
- 2 use in medicine and in surgery.
- 1 7. The artificial human skin according to claim 6 for use on deep, II
- 2 and III-degree burns, for use on various kinds of ulcer selected from
- 3 the group consisting of: diabetic, venous, bedsores, for use in
- 4 plastic surgery and for use in diseases characterized by melanine
- 5 deficiency.

- 1 8. The artificial human skin according to claim 6 for use in genetic
2 therapy, to correct genetic malformations and/or metabolic diseases.
- 1 9. The artificial skin according to one of claims 1-5, as a diagnostic
2 device for *in vitro* tests to verify the possible toxic effect of
3 products and/or substances destined to be placed in contact with the
4 skin and to be used in the chemical, pharmaceutical, cosmetic or
5 agricultural fields.
- 1 10. The artificial skin according to one of the claims 1-5 for use in
2 the cosmetic field for hair grafts.
- 1 11. A controlled release medicament containing the artificial human
2 skin according to one of claims 1-5, as the vehicling agent, said
3 medicament containing at least one active principle selected from the
4 group consisting of biologically active compounds, proteins
5 peptides and mixture thereof, wherein said active principle is
6 adsorbed to the fibres of the non-woven tissues and/or to the
7 microperforated membrane.
- 1 12. The controlled release medicament according to claim 11 wherein
2 said proteins are growth factors, selected from the group consisting
3 of Fibroblast Growth Factors, Transforming Growth Factor β ,
4 Keratinocyte Growth Factor, Nerve Growth Factor, or proteins of the
5 extracellular matrix selected from the group consisting of
6 Fibronectin, Laminin, Collagen, or proteins involved in the clotting
7 cascade.
- 1 13. The controlled release medicament according to claim 12 wherein
2 said proteins of the extracellular matrix are absorbed to the fibres
3 of the extracellular matrix.
- 1 14. A process for preparing the artificial human skin according to one

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2 of claims 1-5 comprising the following steps :

3 i) seeding keratinocytes cultures at a density ranging from 1,000 to
4 100,000 cell/cm², on a microperforated membrane based on a hyaluronic
5 acid derivative and expanding said Kc on said membrane according to
6 conventional techniques involving the use of foetal calf serum,
7 until achieving partial or complete confluence.

8 ii) cultivating the fibroblasts isolated from the dermis or from other
9 districts by the usual techniques in DMEM containing 10% foetal calf
10 serum, conditioning these fibroblasts to proliferate by seeding on
11 plastic, seeding the fibroblasts in the non-woven tissue and
12 continuing the culture until freezing, (in case the obtained
13 artificial skin must be preserved) or until grafting.

14 iii) laying the epithelial layer already formed on the non-woven
15 tissue colonized by fibroblasts, being careful that the outer edge of
16 the microperforated membrane does not touch the bottom of the dish and
17 that it extends slightly beyond the underlying non-woven tissue,

18 iv) optionally fixing together the epithelial cells layer on the
19 microperforated membrane to the underlying non-woven tissue colonized
20 by fibroblasts by means of biological adhyesives selected from the
21 group consisting of: collagen, fibrin, fibrin glue.

22 v) adding a sufficient volume of the same medium used in step (i),
23 keeping the culture immersed so that the keratinocytes of the upper
24 layer of the microperforated membrane are on the air-liquid interface,
25 adding at the first and subsequent changes of medium, ascorbic acid
26 at concentration equal to 1µg/ml, continuing the culture until graft
27 or freezing.

1 15. The process according to claim 14 wherein in step (i) the seeding

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2 density of keratinocytes cells is comprised between 5,000 and 25,000
3 cells/cm², and the cells are expanded in CEC culture medium, in the
4 presence of a "feeder-layer" of nonproliferating 3T3-murine
5 fibroblasts.

1 16. A process for preparing an artificial human skin according to one
2 of the claims 1-5 comprising the following steps:

3 i') seeding the fibroblasts in the non-woven tissue and continuing the
4 culture until freezing or grafting,

5 ii') laying the microperforated membrane on the non-woven tissue, with
6 its edges extending slightly beyond those of the underlying tissue
7 and fixing said membrane with the biological adhesives used in the
8 previous process or by mechanical means,

9 iii') seeding the keratinocytes on the microperforated membranes,
10 expanding said keratynocytes by using said "chemically defined"
11 medium, which is suitable for keratinocyte growth on plastic dishes
12 until achieving confluence,

13 iv') adding a sufficient volume of the same medium used in the
14 preceding step, the culture immersed so that the keratinocytes of
15 the upper layer of the microperforated membrane are on the air-liquid
16 interface, adding at the first and subsequent changes of medium,
17 ascorbic acid at concentration equal to 1µg/ml, continuing the
18 culture until grafting.

1 17. The process according to one of the claims 14 and 15, or the
2 process according to claim 16, wherein, when the skin must be
3 preserved, it further comprises the step of cryopreservation said
4 artificial skin in the presence of a cryopreservation agent.

1 18. The process according to one of the claims 14 and 15, or the

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- 2 process according to claim 16, wherein fibroblasts and keratinocytes
- 3 come from primary cultures or from frozen stocks.

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FIG. 1

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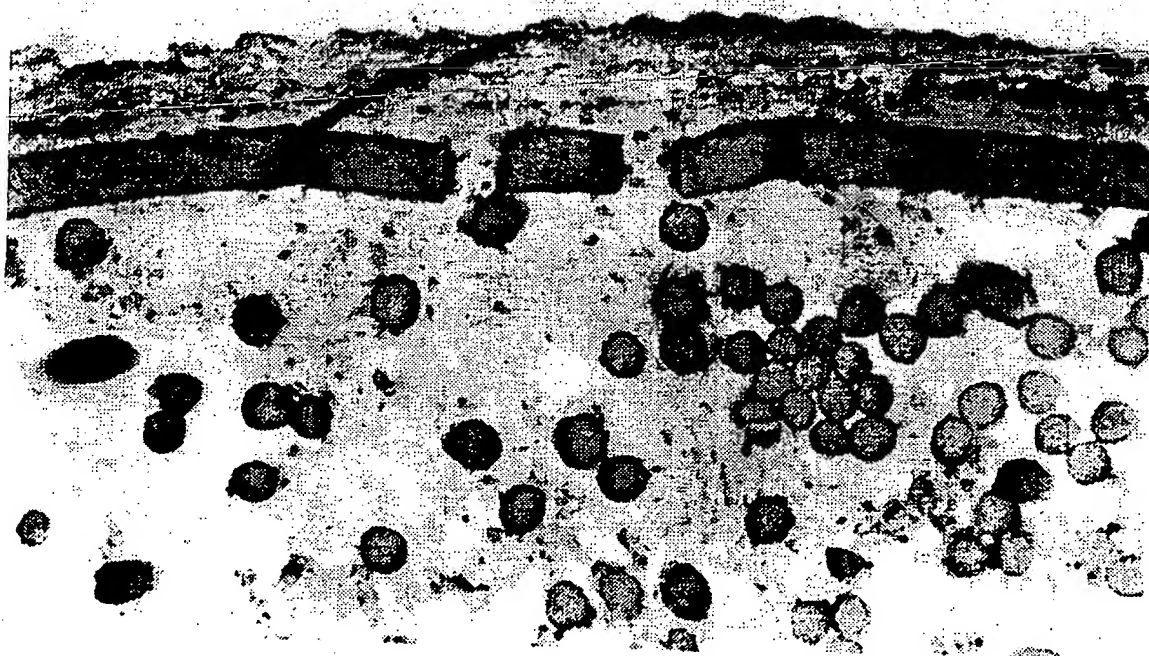


FIG. 2

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FIG. 3



FIG. 4A

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FIG. 4B

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FIG. 5



FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 96/01734

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61L27/00 A61L15/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 462 426 (FIDIA) 27 December 1991 cited in the application see claims 7,11-13,19,20 ---	1-18
A	EP,A,0 265 116 (FIDIA) 27 April 1988 see claim 23 ---	1,6,7
A	WO,A,91 16010 (EISENBERG M.) 31 October 1991 -----	

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

11 September 1996

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Information on patent family members

International Application No

PCT/EP 96/01734

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-462426	27-12-91	IT-B- 1248934	11-02-95
		AU-B- 637235	20-05-93
		AU-A- 7806691	05-12-91
		CA-A- 2043527	02-12-91
		JP-A- 4231061	19-08-92
		US-A- 5326356	05-07-94

EP-A-265116	27-04-88	AU-B- 610087	16-05-91
		AU-A- 7960087	21-04-88
		CA-A- 1317287	04-05-93
		CN-B- 1025035	15-06-94
		FI-B- 96610	15-04-96
		JP-A- 63105003	10-05-88
		NO-B- 175374	27-06-94
		US-A- 4957744	18-09-90
		ZA-A- 8707559	13-04-88
WO-A-9116010	31-10-91	-----	
		AU-B- 632693	07-01-93
		AU-A- 7756991	11-11-91
		CA-A- 2080693	25-10-91
		EP-A- 0526550	10-02-93
		JP-B- 7047043	24-05-95
		US-A- 5282859	01-02-94
